Gentamicin modified chitosan film with improved antibacterial property and cell biocompatibility

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Abstract

Gentamicin modified chitosan film (CS-GT) was produced using a three-step procedure comprising: (i) the chitosan solution was air-dried to form a chitosan (CS) film, (ii) using citric acid to generate the amide and carboxyl groups on the surface of CS, (iii) the CS with surface carboxyl groups was modified by grafting of gentamicin. After modification, this CS-GT film has excellent hydrophilicity and biocompatibility. It is very evident that the gentamicin grafting treatment significantly improves the antibacterial properties of the CS film. Our preliminary results suggest that this novel gentamicin modified chitosan film, which can be prepared in large quantities and at low cost, should have potential application in biomedical applications.

Key words: Chitosan; Gentamicin; Film; Antibacterial property

1. Introduction

To prevent the allergic reaction risks, the surfaces of some metal-base bone substitutes are usually protected by a bioactive organic coating [1-3]. As one of the most representative material

of these coatings, chitosan consists of a large number of hydroxy and amino groups, and possesses outstanding properties, such as non-toxicity, biodegradability and biocompatibility [4-6]. The above advantages make the chitosan-based material has been widely used in the field of tissue engineering [7-8]. However, its application is limited due to the hydrophobic property and common bacterial infection after surgery. Dropping antibiotics to the wound is an efficient method to solve this problem, but it is difficult to fix the amount of the antibiotic, which would cause the waste of the drugs. In addition, it is also a troublesome work to drop the drugs so frequently [9-11].

In this paper, we use the chemical cross-linking method to add gentamicin, a broad-spectrum aminoglycoside antibiotic which is effective against most species of both gram-positive and gram-negative aerobic bacteria through inhibits the bacteria protein synthesis [12-14], to the chitosan to prepare a novel gentamicin-containing antibacterial chitosan film (CS-GT). The gentamicin was grafted onto the surface of chitosan film via citric acid as a transitional object, 1-Ethyl-3-(3-dimethyl aminopropyl) carbodiimide (EDC) and N-hydroxysuccinimide (NHS) as the cross-linker. With this approach, we want to overcome the current problems of chitosan materials that used for biomedical applications.

2. Material and methods

2.1. Materials

Chitosan ($\geq 95\%$ deacetylated) and citric acid were purchased from Aladdin Industrial Inc, (Shanghai, China). Gentamicin sulfate (GT) was purchased from Sigma-Aldrich (St. Louis, MO, USA). EDC was purchased from Sass chemical technology co., LTD, (Shanghai, China) and NHS was obtained from Aladdin Industrial Inc, (Shanghai, China). All cell-culture related reagents were

purchased from Sigma Chemical (St. Louis, MO, USA). Phosphate buffered saline (PBS) was prepared from the tablet form (Calbiochem Corp, Germany).

2.2. Preparation of films

Firstly, the purified chitosan powder was dissolved in 0.01mol L^{-1} acetic acid solution. Gentamicin and the citric acid were respectively dissolved in deionized water with the concentration of 10 mg mL^{-1} . Secondly, chitosan solution was air-dried to form a CS film. Thirdly, the CS film was immersed into citric acid solution to generate the formation of amide and carboxyl group. Afterwards, the film with surface carboxyl groups was transferred to the gentamicin solution containing EDC and NHS (the mass ratio of EDC:NHS:CS-GT is 1:1:6). The cross-linking process was carried out by stirring the mixture for about 4 h. After modification, the CS-GT film was rinsed 3 times with Milli-Q water, and air-dried again. For comparison, chitosan film without chemical modification was prepared as the control group (CS). Scheme 1 displays the schematic illustration of possible reaction routes involved in the formation of CS-GT film.

2.3. Fourier transform infrared (FTIR) spectroscopy

The FTIR spectrum of the CS and CS-GT film was analysed using Fourier transform infrared-attenuated total reflectance (FTIR-ATR, Vector 33, Bruker, Germany). Before acquiring the transmission infrared spectra of the two samples, a background spectrum was collected. All the spectrums were gained obtained from 4000 to 650cm^{-1} .

2.4 X-ray photoelectron spectroscopy (XPS)

X-ray photoelectron spectroscopy (XPS) spectrometer used was a Kratos Analytical (UK) model Axis Ultra system with a mono Al K α X-ray source ($h\nu = 1486.6\text{ eV}$, 150 W). Binding energy was calibrated by C 1s of C–C as 284.6 eV . All spectra were obtained using a $202\text{ }\mu\text{m}$

diameter analysis area. The wide scanning was gathered with pass energy of 140.00 eV and a step size of 0.25 eV, while high-resolution region scans were operated with pass energy of 55 eV at a scan rate of 0.1 eV per step over a range of 1000 eV. Chemical analysis and quantification spectra from the individual peaks of the CS and CS-GT film were obtained. A Gaussian-Lorentzian function was applied to fit the spectra for each peak, in order to assume the kinds and quantifications of the main elements.

2.5. Surface contact angle measurements

Contact angles (CA) were tested by the sessile drop method. Measurement was carried out on Powereach JC2000D1 with an injection volume of 1 μ L distilled water as medium. CA was calculated by a circle segment function intersecting with a straight baseline representing the surface (n=5).

2.6. Swelling test

Swelling studies were performed for CS and CS-GT using PBS (PH=7.4). Water absorption of the CS and CS-GT film was tested by swelling them in PBS at about 37 °C. Wet weight of the films was, after gently blotting the membrane surface with filler paper to remove the absorbed water, weighed immediately at different time intervals. The water absorption of the films was calculated by the following formula:

$$\text{Water absorption} = (M_t - M_0)/M_t \times 100 \%, \quad (1)$$

It's worth noting that the dry materials have swelling feature after absorbing water, so it's meaningful to measure the dimensional changes of the swelled films. The thickness and surface area of the CS and CS-GT films were measured by a micrometer caliper and rules, respectively.

Variations of thickness and surface area are calculated according to the following formulas:

$$\text{Thickness increase} = H_t / H_0; \quad (2)$$

$$\text{Surface area increase} = S_t / S_0. \quad (3)$$

Here, M_0 is the primal dry weight of the film and M_t represents the wet weight of the samples. Meanwhile, H_0 and S_0 are the initial thickness and surface area of the dry samples, respectively. H_t and S_t are the thickness and surface area of the wet samples at target times, respectively. The values are expressed as the means $\pm$ standard error (n=10).

2.7. Assessment of drug release in vitro

Gentamicin release in vitro trials were performed in an incubator under 37°C. 225 milligram CS-GT film was soaked in 15 mL PBS (pH=7.4). 3 mL supernatant of the sample media were collected at regular time intervals (1, 2, 4, 8, 12, 24, 48, 72, 96, 120, 144 and 168 h, respectively). The volume of the medium was maintained at a constant volume by adding phosphate buffer solution equal to the amount withdrawn for analysis. The concentrations of drug were measured using the quantified method described in the reference [14]. The percentage of drug release was calculated by dividing the amount of drug in the release medium by the total gentamicin introduced to the CS-GT film. The experiments for drug release were carried out in triplicates.

2.8. Antibacterial test

For characterization of the antibacterial properties of the samples, staphylococcus aureus and escherichia coli were used for antibacterial tests. The antibacterial experiments were conducted following the published procedures [9]. The concentration of the bacterial suspensions was $3-8 \times 10^8$ colony-forming units (CFU)/mL. The CS and CS-GT sample that have been immersed in PBS for 1, 3, 5 and 7 days were placed into 48 well plates (Corning, UK), covering with 0.5 mL bacteria suspension in logarithmic growth phase and incubated by gentle rotation for 24 hours at

37°C. The viable bacteria in the buffer were quantified by plating serial dilutions on agar plates. Agar plates were incubated for 24 h at 4°C, and the total colony forming units (CFU) were counted visually (n =5).

2.9. The proliferation of MC3T3-E1 osteoblasts

The cytocompatibility of the samples were evaluated using MC3T3-E1 osteoblasts, which were cultured in MEM (GIBCO), supplemented with 10% FBS, 100 U/mL penicillin, 100µg/ mL streptomycin and 0.2% levofloxacin. The cells were incubated in a humidified atmosphere containing 5% CO₂ at 37 °C. Before cell experiments, the films were washed with PBS under aseptic conditions, then sterilized by ultraviolet radiation. The seeded cell density was about 2000 cells cm⁻² and the culture medium was replaced every day. The proliferation activity of the MC3T3-E1 cells on the samples were quantitatively determined by MTT assay (n=10).

2.10. Statistical analysis

All data are shown as mean ± standard deviation. Experiments were analyzed using analysis of variance (ANOVA) to determine the significant differences among the groups. Statistical significance was defined as P< 0.05.

3. Results and discussion

3.1. ATR-FTIR spectra analysis

Fig.1 shows the ATR-FTIR spectrum of the CS and CS-GT film. The main characteristic bands associated with chitosan molecular are detected. Differences between the spectral of the CS and CS-GT film are also evident. As indicated in Fig. 1, by moving from CS toward the CS-GT, the increases in intensity are more pronounced at gentamicin's characteristic peaks, e.g. at 1559 cm⁻¹ (N-H) and 1637 cm⁻¹ (C=O). Furthermore, some of the characteristic absorption bands of

gentamicin are identified for CS-GT film. The FTIR spectra of CS-GT depict characteristic gentamicin absorption bands at 1151 cm^{-1} and 895 cm^{-1} , which are attributed to the C-O-C stretching. The ATR-FTIR result implies that gentamicin has been graft onto the surface of chitosan film successfully.

3.2. X-ray photoelectron spectroscopy analysis

XPS was used to provide the surface chemical information of the resulting films. XPS spectrum of CS and CS-GT is shown in Fig. 2 and the elemental composition analysis is showed in Table 1. When gentamicin was incorporated on the surface of film, the content of oxygen and nitrogen has changed. After chemical grafting reaction, the nitrogen content on the surface of the film increased from 4.55% to 6.68%, while the oxygen content decreased from 30.07% to 27.70%. Correspondingly, the ratio value of N/C increased from 0.696 to 0.102, and that of O/C decreased from 0.46 to 0.42. The increase in percentage content of nitrogen demonstrates that gentamicin is successfully introduced by chemical grafting.

The high-resolution XPS spectra of C 1s were obtained and peak fittings were performed to further investigate the detailed chemical bonding and compositions of CS and CS-GT (Fig. 3). The C1s spectrum of the samples could be resolved into four main component peaks of different carbon species (Fig. 3A and B). The peaks around 284.80 eV, 286.47 eV, 288.07 eV, 289.02eV are assigned to -C-C-, -C-N-, -C-O- and -C=O-, respectively. The atomic percentage and assigned chemical groups for each peak within the region envelopes are listed in Table 2. The ratio of the absorptive peaks area corresponding to -C-N-, -C-O- and -C=O- increased obviously after the introduction of gentamicin. The increases in carboxyl and the new generated amide bond content in the CS-GT film compared with the CS film is because of the cross-linking of chitosan, citric

acid and gentamicin through the condensation polymerization of amine and carboxyl.

3.3. Surface wettability of CS and CS-GT

Variation of contact angle (CA) versus time of the CS and CS-GT film is given in figure 4. Water spreading on the CS sample occurred within 45 s and the CA value decreased from $98.9 \pm 0.5^\circ$ to $89.1 \pm 0.6^\circ$, revealing the hydrophobic surface of the CS film (Fig.4). However, the CA value of CS-GT sample decreased from $82.7 \pm 0.5^\circ$ to $69.8 \pm 0.6^\circ$. The lower CA value observed on CS-GT sample indicates that its surface is polar and more hydrophilic compared to the CS film. As we know, citric acid has three carboxyl groups and gentamicin has five amide groups, successful grafting of these small molecules may introduce the functional groups at the surface of CS-GT film, which results in a higher molecular polarity of the CS-GT film than that of the CS film, so the CS-GT film exhibits a minor contact angle. Therefore, we concluded that the CS-GT's hydrophilicity is better than that of the CS film. In addition, it's worth noting that the surface hydrophilicity can influence the biomineralization process of a bone repair material, so this CS-GT film should have good potential application in bone tissue engineering [15-17].

3.4. Water absorption of CS and CS-GT

Fig.5 displays the water absorption capacity of CS and CS-GT samples. After the specimens had been placed in PBS for 0.5 hour, the water absorption of the two films tended to be constant. Water absorption percentages for CS and CS-GT is about $59.59 \pm 4.41\%$ and $65.66 \pm 1.45\%$, respectively (Fig.5A). The water absorption capacity of CS-GT film is higher than that of CS film. The moisturizing ability of the chitosan-based sample in PBS solution improves with the addition of the small hydrophilic molecules. This may be due to the introduction of citric acid and gentamicin molecular has brought a large number of hydrophilic functional groups on the surface

of CS-GT film.

Furthermore, it's worth noting that the size of a biomaterial for tissue repair should be easy to control because of the sizes of wounds are often different for each patient [18-19]. After the determination of the dimensions of the samples, the variations of thickness and surface area versus time of the CS and CS-GT film are shown in fig.5B and fig.5C. After the samples had been immersed in PBS for 30 min, the dimensions of the specimens tended to be constant ($n = 10$). Experimental results show that the thickness and surface area of the as-synthesized CS-GT film are controlled and repeatable. According to the target wet material's thickness and surface area, we can obtain the dry CS-GT material with the corresponding size easily.

3.5. In-vitro gentamicin release

The concentration of gentamicin was measured in triplicate using a UV3802 ultraviolet–visible spectrophotometer (Shanghai UNICO, China). The average of three measurements was taken as the reported value. The determination of gentamicin release (cumulative release in vitro) of CS-GT film is shown in Fig. 6. The release profile exhibits an exponential tendency and the burst release of gentamicin from CS-GT in the first four hours is about 47% of total gentamicin loading amount, followed by a slower release of the drug took place. Generally, antibiotics are dropped frequently to the wound in the first week after surgery to treat the bacterial infection [9]. From the first day, the sustained release of gentamicin from the CS-GT film manifested as a slow process within a week. On day 7, the total gentamicin released from the CS-GT film has exceeded 96%.

3.6. The antibacterial effect of the CS and CS-GT film

Currently, although surgical operation has high success rate, a serious problem is that the phenomenon of bacterial infection after transplantation of biomaterials is very common [20-21].

Figure 7 shows the results of antibacterial effect of the CS and CS-GT film in 1 week. Compared to the pure chitosan film, the colony forming units of staphylococcus aureus and escherichia coli on Col-GT's agar culture dish are significantly less than that of CS sample (Fig. 7A and B). The results indicate that the CS-GT film has significant good antibacterial effect than the CS film. Figure 7C and D is the quantitative results of anti-bacterial experimental, the CFU on CS-GT's agar culture dish are significantly lower than that of CS film (Fig. 7C and D, n=5). The results suggest that the CS-GT film sterilize the staphylococcus aureus and escherichia coli successfully. In the future, the antibacterial effect of the CS-GT film on other bacteria strains will be further studied.

3.7. The proliferation of MC3T3-E1 on the CS-GT film

Biocompatibility is the essential factor of a new material for its application in tissue engineering [22]. The quantitative analysis of cellular viabilities of the as-synthesized CS-GT film was evaluated using the MTT assay. The proliferation of MC3T3-E1 cells on the CS and CS-GT film at different time points are shown in Fig. 8, both the two samples show increased absorbance value in the first week. The MC3T3-E1 osteoblasts show a great increase in cell viability after three days on the CS and CS-GT samples. However, the absorbance value at CS-GT group is significantly higher than that at the CS group, suggesting the excellent biocompatibility of the resulting CS-GT film. The unique molecular structure of gentamicin allows it combines with protein by chemical bond or hydrogen bond. For this reason, the surface of the CS-GT film is more conducive to the cell-adhesive proteins binding and subsequent cell adhesion, thus accelerate the process of cell proliferation.

4. Conclusions

Surface of chitosan film was successfully modified via gentamicin grafting in this study. FTIR and XPS results confirmed that gentamicin had successfully covered the chitosan film's surface. After modification, this CS-GT film has good hydrophilicity and excellent biocompatibility. It is very evident that the gentamicin grafting treatment significantly improves the antibacterial properties of the chitosan-based film. Our preliminary results suggest that this novel gentamicin modified chitosan film may have potential use in bone tissue engineering.

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Fig.1. The ATR-FTIR spectrum of the CS and CS-GT film.

Fig.2. XPS survey spectra of the samples: CS (a) and CS-GT film (b).

Fig.3. High-resolution XPS spectra of C1s spectra and peak fit analysis for CS (A) and CS-GT film (B). The C1s spectrum at 285 eV can be resolved into four main component peaks.

Fig.4. Variations of contact angle versus time for the CS and CS-GT film. Values are expressed as means $\pm$ standard error (n=5). The wettability and hydrophilicity of the film's surface improves with the increase of solid-liquid contact time.

Fig.5. Water absorption percentages of the CS and CS-GT film (A). Variation of the CS and CS-GT film's thickness (B) and surface area (C) versus time. Values are expressed as the means $\pm$ standard error (n=10).

Fig.6. Cumulative release of gentamicin from CS-GT film, the gentamicin sustained release from the CS-GT film can last for more than one week.

Fig.7. Photographs of agar media with colonies of *S. aureus* and *E. coli* strains after incubation with the CS and CS-GT film (A, B). Bacterial colony is clearly visible in the CS's agar culture dish, the CFU of *S. aureus* and *E. coli* on CS-GT's agar culture dish are significantly less than that of CS (C, D).

Fig.8. The proliferation of MC3T3 cells on the CS and CS-GT film. Values are expressed as the means $\pm$ standard deviation (n=10).

Table1. Surface elemental compositions of chitosan film before and after modification

Sample	C (%)	N (%)	O (%)	O/C	N/C
CS	65.38	4.55	30.07	0.46	0.696
CS-GT	65.62	6.68	27.70	0.42	0.102

Table 2 Relative intensities of the fitted C1s peak of CS and CS-GT film

Sample	Position (eV)	Relative intensity (%)	Possible element state
CS	284.8	62.20	-C-C-
	286.47	32.58	-C-N-
	288.07	4.41	-C-O-
	289.02	0.90	-C=O-
CS-GT	284.8	51.24	-C-C-
	286.45	35.21	-C-N-
	288.02	11.57	-C-O-
	288.94	2.13	-C=O-